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Electrophoretic Analysis of *Tribolium castaneum* After Combined Bioassay with Cinnamon (*Cinnamomum aromaticum*), Turmeric (*Curcuma longa*) and Onion Seed Powder (*Nigella sativa*)

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ABSTRACT
In Pakistan, there are many plants which contain insecticidal agents. The present research deals with the electrophoretic analysis of Tribolium castaneum treated with combined effect of turmeric powder (Curcuma longa), black onion seed (Nigella sativa) and Cinnamon (Cinnamomum aromaticum). The electrophoretic analysis was composed of DNA isolation, RNA isolation, isolation and purification of proteins and their complete visualization and study on agarose gel electrophoresis and polyacrylamide gel electrophoresis (PAGE) respectively. Combined bioassay was done and synergistic effect with high mortality rate was observed at 24 to 48 hours, for adults of Tribolium castaneum. Electrophoretic analysis of the adults proved significant degradation of mRNA and hence protein function and expression.
Key words: *Tribolium castaneum, electrophoretic analysis, degradation, expression, mortality, insecticidal agents*

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INTRODUCTION

Tribolium castaneum is commonly called red flour beetle or "bran bugs" attack the stored grain products and have a long association with human stored food like flour, grains, meal pasta, nuts, spices, seeds, beans, cereals, chocolate, cake mix and also museum specimens [1].*T. casteneum*, the red flour beetle has chewing mouthparts. They are not able to sting but can cause allergic reactions though they are involved in disease transmission the beetle has two types which are both harmful pests of grains, flour and other house items [2]. These are *Tribolium castaneum* and *Tribolium confusum. Tribolium confusum* appears to be very similar to *Tribolium castaneum*, hence the name [3].

In Pakistan Wheat among all the cereals grains, comprises 80% of the staple food and is highly sensitive to the attack by food pests. Modern insecticidal methodology is harmful and has its disadvantage which includes human health hazards and significant residual toxic effects. Thorough and continuous application of such chemicals creates alarming situations of pollution. Also, insect resistance to these chemicals is also developed [4].

Red Flour beetle among all other arthropods are widely controlled using chemical insecticides. Malathion was an ideal choice but extensive and universal use of insecticides and pesticides has raised the norms of many changes of biochemical as well as physiological origin, leading to insect resistance. Pyrethroid is considered as one of the most effective and efficient insecticide against pests and has been widely used across China [5]. Ketoalcoholic ions present in pyrethroic acids exhibit insecticidal qualities in addition to chrysanthemic. This pesticide containing acids which being strongly lipophilic, affect the central nervous system of the insects. They penetrate rapidly inside the insect on which the pesticide is applied against [6].

Many botanical herbs also help to eradicate pests.Bio- pesticides have been a lot famous among farmers and other agricultural workers for the past years and are greatly in use to solve all above issues [7-9]. Natural plant pesticides are useful for storing processed food safely [10]. Biopesticides being environmentally friendly are being favored as they eliminate the hazardous effects that are caused by chemical insecticides and other sprays and fumigants. These dangers include the risks of poisonous

residues, genetic resistance development, increased pollution and greater price of application [11]. Due to these issues, chemical insecticides are widely replaced by bio-pesticides that are now in high demand [12].

Cinnamon is used as a cure for many diseases including cold, skin diseases, allergic responses and stomach diseases [7]. Studies reveal that cinnamon oil has antimicrobial activity [13].Its hypoglycemic potential, antimicrobial, anti-ulcer and anti-oxidant properties make it a suitable choice to control Red Flour beetle.

Turmeric is a long perennial plant of Ginger family. Plants are gathered for their rhizomes and are often used as dried yellow powder as a common spice. It is assumed to be helpful in the treatment of Cancer, Alzeihmer's disease, allergic reactions and similar other chronic diseases. Turmeric consists of tumerones and artumerones [14]. *C. longa* rhizome has been screened and its Petroleum ether extract has been documented as repellent to *Tribolium castaneum* [15]. It has insecticidal response against *Plutella xylostella* [16]. Turmeric powder is documented several types as one of excellent repellent against *C. chinensis, T. castaneum* [17] and many populations of *S. oryzae* [18].

Nigella sativa commonly called as black onion seed or kalongi belongs to the family ranunculaceae. This spice is native to Europe, South and west Asia and North Africa. It has generally been used as a medicinal plant since ancient times. Olefinic and Oxygenated monoterpenes are the active components of black onion seed principally thymoquinine, p-cymene and thymohydroquinine (Lewinsohn*et al*., 2012). Utilization of these ancient plants to get rid of fleas, mosquitoes and body insects have been referred by many medical practitioners called Al-Qazwini [19].

MATERIAL AND METHODS

Insect rearing and collection

Tribolium castaneum culture samples were gathered from various parts of Lahore. The larvae were reared on a specific diet consisting of semolina and 10% yeast extract. For this purpose a glass jar was taken and 250 g of diet along with 20 adult pairs were introduced in it and kept at 30^{o}C in the cabinet of the lab. The jar was covered with a white muslin cloth. Bioassay analysis was conducted on newly emerged adults and third instar larvae.

Insect Bioassay

The toxicity of turmeric powder, onion seed powder and cinnamon powder was checked in form of combination. The doses and concentration of bioassay were adjusted after treatment and mortality observed for the highest dose. The biological assay was repeated three times and conducted in a triplicate manner. Mortality was observed and recorded after three days and LC_{50} was analyzed by probit analysis SPSS programme. After that adults of highest dose were isolated for electrophoretic analysis.

Control Group

Control group was comprised of optimum temperature of 28°C and 70% relative humidity (RH) in incubator. 30 newly emerged adults were placed in glass vials along with the selected diet that consists of 0.2g of yeast extract and finely grounded 0.8g of semolina. It was done similar to the treated samples. The procedure was repeated for the second instar larvae biological assay. No mortality was observed in the control samples; hence they were compared with the treated or experimental samples.

Sample Collection of Turmeric, Cinnamon and Onion Seed Powders

Fresh leaves, rhizomes and seeds of turmeric, cinnamon, and onion seed were purchased, and then they were grinded to make a fine powder. Single combined concentration was prepared in glass vial after mixing an equal quantity of 10 g of turmeric powder, cinnamon powder and onion seed powder. The selected concentration of turmeric powder, cinnamon powder, and onion seed powder was 0.75g which was then mixed as per gram diet of red flour beetle.

Combined Bioassay

The selected concentration of turmeric powder, cinnamon powder, and onion seed powder i-e 0.75g was mixed as per gram diet which consists of 0.8 g semolina and 0.2 g of yeast extract, of red flour beetle. 10 adults were placed in each vial. In combination of dose, 30 larvae were used in total. The bioassay performed for larvae was conducted in triplicate form and mortality rate was observed at regular intervals after 24 hrs for one week (seven days).

Electrophoretic Analysis

DNA Isolation and visualization on agarose gel electrophoresis

DNA isolation was carried with DNAzol® product following the user guide provided by the MRC, Inc [20]. The samples were homogenized and lysed using DNAzol which leads to the precipitation of genomic DNA when washed with ethanol. After the completion of ethanol wash, DNA is solubilized in 8mM NaOH or distilled water. The whole procedure was successfully performed and completed in 15 - 40 minutes with

a genomic DNA recovery of 65-95%. The isolated DNA was further analyzed using standard procedure agarose gel electrophoresis consisting of 0.8% agarose gel.

RNA Extraction and Visualization on agarose gel electrophoresis

RNA was extracted using commercially available TRI Reagent® and following the user guide manual by MRC, Inc [20]. The tissue samples were homogenized and then lysed using the reagent. Addition of chloroform and centrifugation facilitates the separation of homogenate in three different layers comprising of aqueous and organic phases. RNA is present in the aqueous phase and is precipitated by the addition of isopropanol and later washed by ethanol and solubilized whereas organic phase comprises of proteins. DNA exclusively remains in the interphase. The entire procedure was completed in 1 hour. High quality of RNA is yielded using TRI reagent and it can isolate RNA from a vast variety biological material, including plant and animal tissues. The isolated RNA was further visualized and analyzed on agarose gel electrophoresis using standard procedure of 0.8% agarose.

Protein Isolation and Analysis through Polyacryamide Gel Electrophoresis (PAGE)

Proteins were extracted using commercially available TRI Reagent®and following the user guide manual by MRC, Inc [20]. Finely grounded tissue sample is homogenized with the help of TRI Reagent as previously done for RNA isolation. Addition of TRI Reagent followed by chloroform and centrifugation for 5 minutes allows the formation of three distinct layers in which proteins is present exclusively in the organic phase. RNA is present in the aqueous layer whereas DNA in the interphase. A part of the supernatant of phenol-ethanol (0.2 - 0.5 ml, 1 volume) was aliquot in a new eppendorf. Three volumes of acetone are added to separate the protein components and tubes were inverted for 15-18 seconds to form a uniform mixture. Samples were stored for 15 min at 37^{o}C and the precipitated protein was sediment after centrifugation at 12,000 rpm for 8 min at 4^{o}C.The pellet was dispersed and washed in 0.5 ml in 95% ethanol. Wash solution was decanted and wash was repeated twice by addition of 1 ml of ethanol wash solution each time. Alcohol was decanted by inverting the microfuge tube and drying the pellet for 8-12 min at room temperature. Air-drying the pellet for a short span, 0.2 ml of 1% SDS is added as a solvent and to assist in solubilization per 15-25 mg of tissue sample. The pellet was gently dispersed and solubilized. The solubilized proteins were stored at -20^{0}C. Proteins were analyzed using standard PAGE procedure using 10% resolving and 5% stacking gel.

RESULTS

The Adult bioassay was performed similar to larvae bioassay. The mortality of *T. castaneum* adult was also observed after 1st day of treated samples and was seen high then the rate decreased as the days progressed and the mortality was increased as the concentration of combined powder of turmeric, onion seed and cinnamon was increased. At 0.75g mortality was found to be 10 of 30. The mortality of *T. castaneum* adult was found high at end of day 4 of treated samples whereas day 5, 6 and 7 was less in comparison. In first three days mortality was high and increased as concentration doses of combination powder were increased.

Electrophoretic Analysis of *Tribolium castaneum*

Electrophoretic Pattern of DNA Bands

The isolated DNA of both the treated and control samples were analyzed by running on agarose gel along with the molecular markers and compared with each other (Fig 1).The DNA of controlled samples showed dense complete patterns. The treated samples showed less dense patterns. Also the DNA band for the controlled sample was clearly visible and that of the treated sample appeared faint and was not compact. This indicated the insecticidal effect caused by the combined concentration of turmeric powder, onion seed powder and cinnamon powder on adults by affecting the DNA band pattern.

Electrophoretic Pattern of RNA

RNA was isolated and run on agarose gel to check the electrophoretic pattern of both the controlled and treated samples along with the molecular markers. Two control samples were run, there electrophoretic pattern was clearly divided into a rod like appearance called mRNA with two distinct bands of tRNAand rRNA at the bottom.The treated samples appeared blurred showing a single faint rod with no clear tRNA and rRNA. This indicates the interference of the selected dose with protein synthesis and protein expression because of mRNA degradation of the organism proving the insecticidal effect. It was confirmed by PAGE analysis of proteins (Fig 2).

Electrophoretic Pattern of Proteins

Protein extracts were analyzed by polyacrylamide gel electrophoresis (PAGE). The PAGE patterns of the controlled samples appeared as dark distinct bands indicating various different proteins of relative molecular weights. The treated samples showed almost complete disappearance of protein bands that were clearly visible in control samples. A reduction in the size of protein band 3 of 68 kDa was also

observed which had large band size in control samples and it proved that combined concentration has insecticidal effect and has interfered in protein function and expression.

DISCUSSION

The present research study was conducted on the combined pesticidal effect of Cinnamon powder (*Cinamomum aromaticum*), turmeric powder (*Curcuma longa*) and onion seed powder (*Nigella sativa*) on *Tribolium castaneum*. On first day of treatment the mortality of insect was high but it gradually decreased as days progressed. The adult of *T. castaneum* of highest dose i-e 0.75g per 1g of feed were used for electophoretic analysis. The electrophoretic analysis included DNA, RNA and protein analysis of the treated and controlled samples.

The DNA was isolated from both the controlled samples and samples treated with the combined dose. The isolated DNA of both the samples was run on agarose gel along with the molecular markers. The DNA of controlled samples showed dense complete patterns. The treated samples showed less dense. Also the DNA band for the controlled sample was clearly visible and that of the treated sample appeared faint and was not compact. This indicated the insecticidal effect by affecting the DNA band pattern.

Two control samples were run, for RNA analysis, there electrophoretic pattern was clearly divided into a rod like appearance called mRNA with two distinct bands of tRNA and rRNA at the bottom whereas treated samples appeared blurred showing a single faint rod with no clear tRNA and rRNA. This indicates the interference of the selected dose with protein synthesis and protein expression of the organism proving the insecticidal effect of the combined concentration of turmeric powder, onion seed powder and cinnamon powder that was of 0.75g .The blur appearance of mRNA clearly indicated that the mRNA is degraded and that the combined dose interferes in the protein synthesis and expression.

Protein extracts were analyzed by polyacrylamide gel electrophoresis (PAGE). The PAGE patterns of the controlled samples appeared as dark distinct band indicating various different proteins of relative molecular weights. However, a major disappearance of all the protein bands were observed indicated that the combined concentration has insecticidal effect and has interfered in protein function and expression. The treated samples showed almost complete disappearance of protein bands that were clearly visible in control samples.

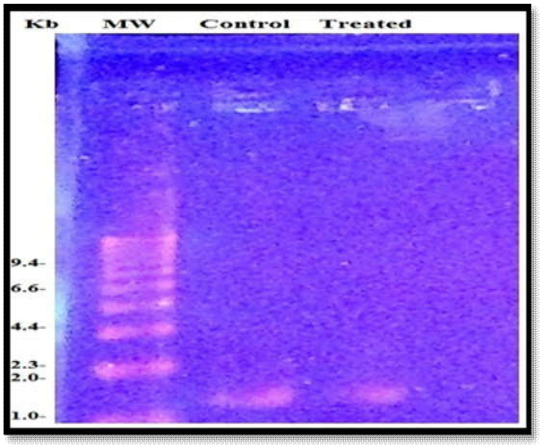

Fig 1 : Agarose gel patterns of DNA of adults of control and treated samples

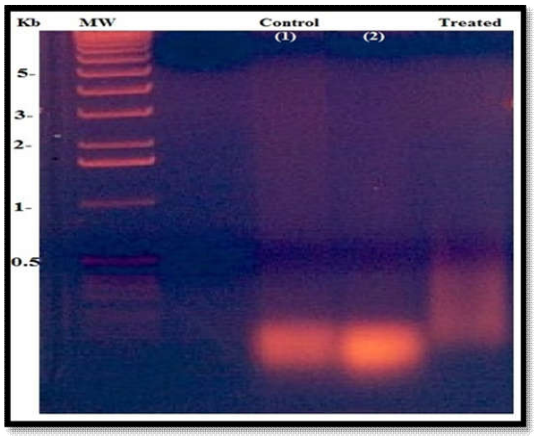

Fig 2: Agarose Gel Patterns of RNA of Adults of Control and Treated Samples

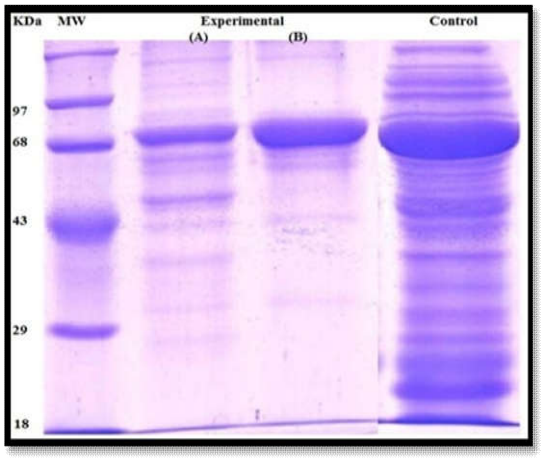

Fig 3 PAGE Patterns of Protein Extracts of Adults of Control and Treated Samples

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